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The influence of smoking and diet on the hypoxanthine phosphoribosyltransferase (*hprt*) mutant frequency in circulating T lymphocytes from a normal human population

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Abstract

The influence of the dietary antioxidants vitamin C, α- and β-carotene, lycopene, lutein/zeaxanthin, phytofluene, β-cryptoxanthin, retinol and α- and γ-tocopherol on the hypoxanthine phosphoribosyltransferase (*hprt*) mutant frequency in human peripheral T lymphocytes was investigated. Twenty-five male non-smokers and 27 male smokers in the age range 50–59 years were recruited. Smokers showed a significantly higher mutant frequency compared with non-smokers ($\times 1.5$, $P < 0.01$). In addition, there was a significant positive relationship between *hprt* mutant frequency and the number of cigarettes that individuals reported smoking daily ($P < 0.01$). Smokers showed significantly lower levels of plasma vitamin C and the carotenoid α-carotene than non-smokers ($P < 0.01$ and $P < 0.05$ respectively). Both *hprt* mutant frequency and lymphocyte plating efficiency were weakly inversely associated with plasma vitamin C levels ($P < 0.07$ and $P < 0.06$ respectively) suggesting that vitamin C may be protective against mutation at the *hprt* locus. This relationship was markedly stronger in smokers ($P < 0.01$).

Keywords: Hprt mutant; Human lymphocytes; Antioxidant; Diet; Biomarker

1. Intoduction

Reactive oxygen species generated in vivo during normal aerobic respiration and metabolism are involved in the pathogenesis of a wide variety of diseases and degenerative conditions such as cardiovascular disease, rheumatoid arthritis, cataracts, ageing and cancer (Cross et al., 1987; Duthie et al., 1989; Duthie, 1991; Halliwell et al., 1992). Moreover, the body is exposed to free radicals from exogenous sources such as environmental contaminants. Cellular defence mechanisms have evolved; the tripeptide glutathione, glutathione peroxidases, catalase and superoxide dismutases inactivate these deleterious oxygen species and radicals before they damage critical cellular macromolecules such as DNA and lipids. In addition, there are enzymatic pathways which subsequently repair the small amount of DNA damage which inevitably occurs. Antioxidant micronutrients such as the free radical scavengers vitamin E, carotenoids, vitamin C and flavonoids

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are obtained from the diet. To date, evidence for a significant effect of these micronutrients in protecting against cancer in the human population is based on extensive epidemiological studies (Miller, 1990; Stahelin et al., 1991; Byers and Perry, 1992). There is strong evidence for a protective effect of carotenoids, particularly β-carotene, against lung cancer and possibly stomach cancer in smokers (Connet et al., 1989; Ziegler, 1989; Dorgan and Schatzkin, 1991). Low vitamin C intake is associated with an increase in the risk of oral cancer and cancer of the oesophagus, larynx, pancreas and stomach (Dorgan and Schatzkin, 1991; Block, 1991) and while evidence supporting an anticarcinogenic role for vitamin E is generally weak, moderately protective effects against oesophageal, gastrointestinal and bladder cancer have been reported (Dorgan and Schatzkin, 1991; Knekt et al., 1991a,b). Conventional epidemiological studies suggest a connection between diet and cancer but reveal nothing about the biochemical processes involved in the anticarcinogenic action of dietary antioxidants.

The complementary approach of molecular epidemiology measures well defined biomarkers at the molecular level without relying on subsequent development of disease and should provide information on the mechanisms by which these dietary antioxidants protect. Such an approach has been adopted in the present study.

We have measured the frequency of mutations in the hypoxanthine phosphoribosyl guanine transferase (*hprt*) gene in human lymphocytes as a biomarker for genetic change and have related it to plasma antioxidant levels. Mutations in this gene are widely used in population studies as a marker reflecting long-term exposure to mutagenic influences (Cole and Skopek, 1994). Hprt is a non-essential salvage enzyme which recycles purines into the cell's DNA biosynthetic pathways. Mutation of the *hprt* gene is not lethal. The frequency of $hprt^-$ mutants is determined by exposing lymphocytes to the toxic purine analogue 6-thioguanine (6-TG), which is selectively taken up by normal $hprt^+$ cells and kills them. Conversely, $hprt^-$ mutants grow in the presence of 6-TG and mitogen. If dietary antioxidants are protective against DNA damage in vivo we would expect to find an elevated $hprt^-$ mutant frequency in individuals with low antioxidant levels. This work is part of a larger double-blind, strictly controlled intervention trial which has been described previously (Collins et al., 1994).

2. Materials and methods

L-Glutamine, mitomycin C, penicillin G, pyruvic acid (α-ketopropionic acid, sodium salt), streptomycin sulphate and 2-amino-6-mercaptopurine (6-thioguanine) were from Sigma (Poole, UK). All Nunc sterile tissue culture plastics were supplied by Gibco Life Technologies Inc. (Paisley, UK). Lymphoprep was supplied by Nycomed UK (Birmingham, UK), and Dutch Modified RPMI 1640 medium containing 20 mM Hepes buffer was from ICN Flow (Irvine, UK). Murex Diagnostics Ltd (Dartford, UK) provided HA15 phytohaemagglutinin while recombinant interleukin (Aldesleukin 18×10^6 IU) was purchased from EuroCetus UK Ltd (Harefield, UK). Ventrex HL-1 serum-free medium was from New Brunswick Scientific Biologicals (Hatfield, UK), and foetal calf serum (batch 141) was from Globepharm Ltd (Surrey, UK).

GM 1899A lymphoblastoid cells were a generous gift from Dr. Jane Cole, MRC Cell Mutation Unit, University of Sussex, Falmer, Brighton, UK.

Selection of subjects and blood sampling

Blood was collected from normal healthy males aged between 50 and 59 years. Individuals on drug therapy or health supplements or with a history of heart disease were excluded. Twenty-seven smokers and 25 non-smokers were recruited. Non-smokers were defined as those who reported never having smoked; ex-smokers were not used in this study. Each volunteer completed a questionnaire designed to give details of past and present health status and exposure to potential mutagens. Blood collected from the volunteers was used to determine *hprt* mutant frequency and the plasma antioxidants vitamins C and A, α- and γ-tocopherol and carotenoids. Cotinine, a breakdown product of nicotine, was measured in urine by gas chromatography as an

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indicator of smoking status (Beckett and Triggs, 1966).

Isolation of lymphocytes for hprt assay

Individual venous blood samples (50 ml) were collected into vacutainers containing 143 units of lithium heparin and the lymphocytes immediately isolated at room temperature as follows: blood was diluted 1:1 with Dutch Modified RPMI tissue culture medium (referred to below as medium) before layering onto an equal volume of Lymphoprep lymphocyte separation medium (specific gravity 1.077 ± 0.001 g/ml) and centrifuging at $700 \times g$ for 30 min. The mononuclear cells (buffy coat) were removed into fresh centrifuge tubes, washed using medium and spun for a further 20 min at $700 \times g$. The supernatant was decanted, the cell pellets from one individual pooled and washed in medium, spun at $700 \times g$ for 15 min and finally resuspended in medium containing 2 mM L-glutamine, 100 μg/ml pyruvic acid, 100 U/ml penicillin and 100 μg/ml streptomycin and 10% foetal calf serum before being counted using a Neubauer Improved Haemocytometer. The cells were subsequently pelleted by centrifuging at $700 \times g$ for 15 min and resuspended at 3×10^6 cells/ml in medium containing 10% foetal calf serum, 40% HL-1 serum-free medium, 2 mM L-glutamine, 100 μg/ml pyruvic acid, 100 U/ml penicillin and 100 μg/ml streptomycin. The mononuclear cells were incubated in this complete medium at 37°C in 95% air/5% CO_2 overnight (18–20 h).

Incubation of GM1899A cells with mitomycin C

GM1899A cells were grown as suspensions at 37°C in 95% air/5% CO_2 in Dutch Modified RPMI 1640 medium containing 10% foetal calf serum, 2 mM L-glutamine, 100 μg/ml pyruvic acid, 100 U/ml penicillin and 100 μg/ml streptomycin. Feeder cells for the *hprt* mutation assay were prepared by incubating actively growing GM1899A cells at approximately $1-2 \times 10^5$ cells/ml with 10 μg/ml mitomycin C in DMSO for 16 h at 37°C in 95% air/5% CO_2. These drug-treated cells were washed thoroughly 3 times in medium before being used in the *hprt* mutation assay. Treatment with mitomycin C at this concentration immediately inhibited GM1899A cell growth and caused 100% cell death after a further 3 days in culture (data not shown).

Lymphocyte cloning

Lymphocytes were cloned essentially after the method of Henderson et al. (1986) and Cole et al. (1988). To minimise the influence of experimental factors on the assay, single batches of heat-inactivated foetal calf serum, phytohaemagglutinin and interleukin were used. The ability of the foetal calf serum to support lymphocyte growth was established beforehand in bulk suspension cultures. All cloning assays were carried out by the same laboratory worker.

After the overnight culture period non-adhering lymphocytes were counted as before and incubated at a final cell density of either 3 cells/well in 2×96 well flat-bottomed tissue culture plates to determine the plating efficiency of the cells or at 2×10^4 cells/well in the presence of 10 μM 6-thioguanine in a maximum of 8×96 well flat-bottomed plates to estimate mutant frequency.

All cells were incubated at 200 μl/well in Dutch Modified RPMI 1640 medium supplemented with 10% foetal calf serum, 40% HL-1 serum-free medium, 2 mM L-glutamine, 100 μg/ml pyruvic acid, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5% phytohaemagglutinin, 100 units/ml interleukin and mitomycin C-treated feeder cells at a concentration of 5×10^4/ml. The plates were taped to minimise dehydration and incubated at a 5% slope at 37°C under 95% air/5% CO_2 for 14–17 days before being scored for cell growth using a Hund Wetzlar phase contrast inverted microscope. Wells scored as positive contained colonies of more than 50 cells.

To determine the variation within the assay, blood from a single individual was assayed every 6–8 weeks throughout the 9-month sampling period and the plating efficiency and mutant frequency calculated.

Analysis of data

The cloning efficiency (CE) for the selective (6-thioguanine containing) and non-selective plates was calculated from the following formulae after the method of Henderson et al. (1986):

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Table 1
Hprt⁻ mutant frequencies in blood samples from volunteers

Subject	Cloning efficiency (CE)	P(0) selective plates	Mutant frequency ($\times 10^{-6}$)
1	17.38	709/768	22.9
2	37.40	565/660	20.79
3	11.26	738/768	17.69
4	49.1	545/768	34.93
5	17.08	652/768	47.93
6	16.5	661/768	45.47
7	59.73	564/768	25.84
8	16.9	755/768	5.05
9	75.4	503/768	28.06
10	7.14	591/614	26.74
11	14.04	637/672	19.05
12	6.4	700/768	72.43
13	17.2	715/768	20.79
14	72.2	746/768	2.01
15	17.97	665/768	40.07
16	89.76	592/768	14.49
17	40.48	631/768	24.26
18	11.01	738/768	18.09
19	48.36	585/672	14.33
20	70.73	600/768	17.45
21	55.79	365/480	24.55
22	55.80	690/768	9.59
23	72.2	394/768	46.22
24	61.87	600/768	19.94
25	72.22	578/768	19.68
26	89.75	532/768	20.45
27	92.42	554/768	17.67
28	18.3	710/768	21.45
29	28.3	534/560	8.39
30	18.6	724/768	15.86
31	30.9	730/768	8.21
32	50.66	328/384	15.56
33	36.6	446/480	10.04
34	49.1	401/468	15.73
35	21.7	714/743	9.17
36	18.6	733/768	12.54
37	27.56	697/768	17.59
38	22.8	727/768	12.03
39	20.99	695/768	23.79
40	10.76	725/768	26.77
41	21.41	682/768	27.73
42	13.78	753/768	7.16
43	12.99	732/768	18.48
44	19.89	723/766	14.52
45	22.41	559/575	6.29
46	14.31	720/768	22.55
47	41.07	475/576	23.47
48	9.1	709/768	43.92
49	43.54	637/768	21.48
50	65.38	670/768	10.44
51	115.52	678/768	5.39
52	59.72	640/768	15.26

CE = − ln (proportion of negative wells)
/number of mononuclear blood cells per well

Mutant frequency (MF) = CE in selective plates/CE in non-selective plates

Analysis was performed using the statistical package 'Genstat'. No data were excluded before analysis but to prevent undue weight being given to outlying points, mutant frequencies and plating efficiencies were log transformed before performing correlation analysis. In the case of mutant frequency and plating efficiency related to vitamin C, since the data are clearly not normally distributed, a ranking test was deemed most appropriate.

Plasma antioxidants

Plasma was prepared from heparinised whole blood (10 ml) by centrifuging at $2000 \times g$ for 15 min at 4° C. The samples were immediately 'snap frozen' using liquid nitrogen and stored at −80° C. All plasma was prepared and frozen within 1 h of blood collection. Where samples were prepared for ascorbic acid determination an equal volume of 10% (w/v) metaphosphoric acid was added to the plasma before freezing. Ascorbic acid concentration was determined by the method of Ross (1994). Plasma concentrations of retinol, α-tocopherol and γ-tocopherol and the carotenoids α-carotene, β-carotene, β-cryptoxanthin, lycopene, phytofluene, and lutein/zeaxanthin were measured after the method of Hess et al. (1991). Plasma antioxidant values reported here are the means of 3–4 samples taken over a 20-week period.

3. Results

Hprt mutant frequency data were collected from single blood samples from 52 healthy male

Notes to Table 1:
Recruits were males aged 50–59 years. Samples 1–27 were collected from smokers and samples 28–52 from non-smokers. All non-selective plates were set up at 3 cells/well and at 2×10^4 cells/well in selective plates where 6-thioguanine (10 μM) was included in the medium. CE refers to the cloning efficiency of the lymphocytes while P(0) is the number of negative wells/number of wells plated in the selective plates.

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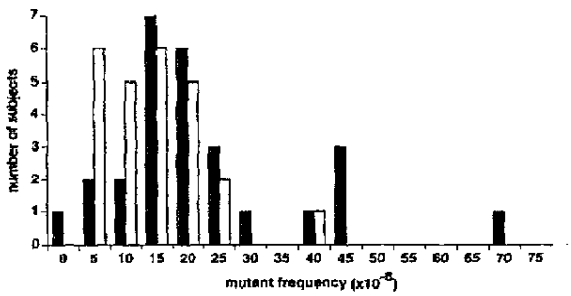

Fig. 1. Comparison of *hprt* mutant frequencies in smokers and non-smokers. Open blocks represent non-smokers (n = 25), while shaded blocks represent smokers (n = 27).

volunteers aged between 50 and 59 years (Table 1). The mutant frequencies for these individuals ranged between 2.01×10^{-6} and 72.4×10^{-6}, with a population mean of $20.96 \pm 1.79 \times 10^{-6}$ (Fig. 1).

Mutant frequency was elevated 1.5-fold in lymphocytes isolated from smokers compared with non-smokers (Table 2). There was no difference in the mean age of individuals in the two groups (53.7 ± 0.5 years for smokers and 55.4 ± 0.5 years for non-smokers). There was a significant positive relationship between mutant frequency and the

Table 2
Hprt⁻ mutant frequencies and plasma antioxidant levels in smokers and non-smokers

	Smokers	Non-smokers
Mutation frequency	25.2 ± 2.9 (27) * *	16.6 ± 1.7 (25)
Cotinines	2.11 ± 0.16 (27) * *	0.04 ± 0.01 (25)
Vitamin C	23.96 ± 4.32 (17) * *	41.15 ± 2.31 (21)
α-Tocopherol	5.01 ± 0.13 (17)	5.13 ± 0.15 (21)
γ-Tocopherol	0.34 ± 0.03 (17)	0.38 ± 0.03 (21)
α-Carotene	0.043 ± 0.014 (17) *	0.059 ± 0.028 (21)
β-Carotene	0.246 ± 0.014 (17)	0.297 ± 0.026 (21)
Retinol	0.549 ± 0.032 (17)	0.538 ± 0.021 (21)
Lycopene	0.312 ± 0.038 (17)	0.241 ± 0.029 (21)
β-Cryptoxanthin	0.055 ± 0.008 (17)	0.061 ± 0.007 (21)
Phytofluene	2.235 ± 0.459 (17)	1.771 ± 0.282 (21)
Lutein/ zeaxanthin	3.842 ± 0.365 (17)	3.978 ± 0.349 (21)

Results are mean ± SEM for each group.
* $P < 0.05$, * * $P < 0.01$. Significance refers to differences between smokers and non-smokers measured by Student's unpaired *t*-test.

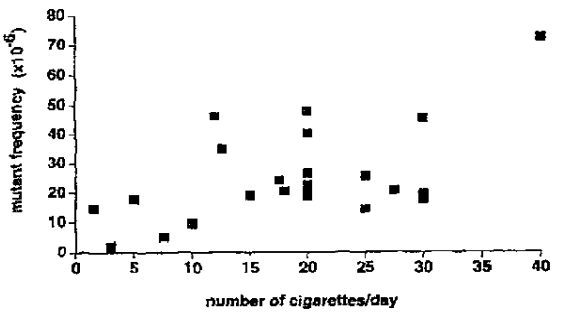

Fig. 2. Cigarette intake and *hprt*⁻ mutant frequency in smokers. Each data point represents the mutant frequency and cigarette intake for one individual. Daily cigarette consumption was derived from a lifestyle questionnaire (n = 24, $r = 0.579$. $P < 0.003$).

number of cigarettes that individuals reported smoking daily (Fig. 2: $r = 0.579$, $P < 0.003$). This was also seen when mutant frequency and cotinine levels were compared ($r = 0.376$, $P < 0.05$), together with a highly significant correlation between cotinine levels and cigarette intake for smokers (Fig. 3: $r = 0.651$, $P < 0.0006$).

There was a significant inverse relationship between plating efficiency and mutant frequency for the population as a whole ($r = -0.273$, $P < 0.05$).

Smokers had significantly lower levels of vitamin C and α-carotene than non-smokers (Table

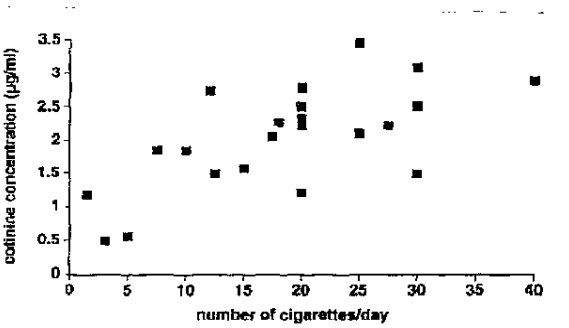

Fig. 3. Cigarette intake and cotinine levels in smokers. Cigarette intake was derived from a lifestyle questionnaire and cotinine levels were measured by gas chromatography as described in the methods section (n = 24, $r = 0.651$, $P < 0.0006$).

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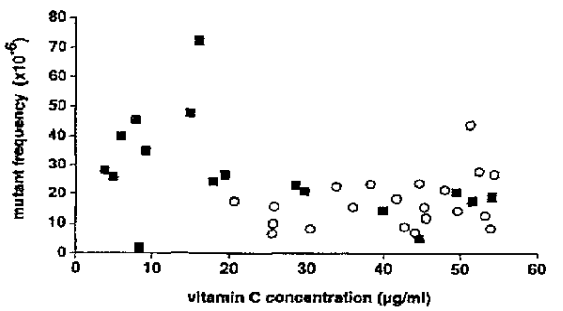

Fig. 4. *Hprt*⁻ mutant frequency and plasma ascorbic acid levels. Each data point represents the mutant frequency and mean plasma ascorbic acid level for one individual (■ = smokers, n = 27; ○ = non-smokers, n = 25; $r = -0.295$, $P < 0.07$).

2). There was a weak association between both mutant frequency and plating efficiency and plasma vitamin C levels for the population as a whole (Figs. 4 and 5: $r = -0.295$, $P < 0.07$ and $r = -0.310$, $P < 0.06$ respectively). The relationship between mutant frequency and vitamin C levels proved much stronger in the smokers ($r = -0.592$, $P < 0.01$).

Plasma levels of α-tocopherol, γ-tocopherol, α-carotene, β-carotene, β-cryptoxanthin, phytofluene, lutein/zeaxanthin and retinol were similar in smokers and non-smokers (Table 2)

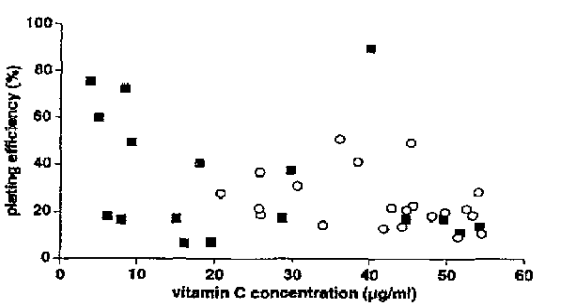

Fig. 5. Lymphocyte plating efficiency and plasma ascorbic acid levels. Each data point represents lymphocyte plating efficiency and mean plasma ascorbic acid level for one individual (■ = smokers, n = 27; ○ = non-smokers, n = 25; $r = -0.310$, $P < 0.06$).

and were not significantly related to mutant frequency.

The plating efficiency (mean, 29.9 ± 3.5% and range, 20.5–37.1%, n = 4) and mutant frequency (mean, $15.23 \pm 1.16 \times 10^{-6}$ and range $11.8–16.6 \times 10^{-6}$, n = 4) of the standard blood samples used to estimate the variation within the assay were well within the accepted values for repeat blood samples from the same individual as reported by Cole and Skopek (1994).

4. Discussion

The occurrence of T-lymphocytes resistant to the toxic purine analogue 6-thioguanine is regarded in human population monitoring as an indicator of somatic mutations in vivo. *Hprt*⁻ mutant frequencies have been measured in normal healthy populations (O'Neill et al., 1987; Cole et al., 1988,1990,1991; Tates et al., 1991; Davies et al., 1992), in individuals exposed to radiation, chemotherapeutic drugs, or cytotoxic chemicals (Sala-Trepat et al., 1990; Caggana et al., 1991; Branda et al., 1991,1993; Tates et al., 1994a,b) and in individuals with inherited DNA repair defects (Cole et al., 1992). Variability in mutation frequency at the *hprt* locus in populations of normal individuals is large, ranging over two orders of magnitude (Cole and Skopek, 1994), and is influenced by a number of factors. *Hprt*⁻ mutant frequency is generally 7–10-fold higher in adults than in newborns and increases further at a rate of 1–5% per year in adults (Cole et al., 1988,1990,1991; McGinnis et al., 1990; Tates et al., 1991; Branda et al., 1993). The mean mutant frequency in our population is higher ($20.96 \pm 1.79 \times 10^{-6}$) than that reported by many laboratories and this may reflect the age of our subjects. In support of this, Tates et al. (1991) reported a mean population *hprt* mutant frequency of 22.4 $\pm 5.40 \times 10^{-6}$ for a group of cancer patients (before radiation treatment) with an average age of 55.5 ± 2.42 years.

Gender appears not to influence *hprt*⁻ mutant frequency (Cole et al., 1988,1990; Branda et al., 1993), although Caggana et al. (1991) investigat-

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ing therapy-related effects on in vivo somatic mutations in Hodgkin's lymphoma patients found a significant sex effect in the control population.

Hprt$^-$ mutant frequency generally increases with decreasing plating efficiency (Cole et al., 1988,1990,1991; Tates et al., 1991; Branda et al., 1993) although exceptions to this have been reported (Davies et al., 1992). In our study a significant correlation between mutant frequency and plating efficiency was observed.

Cigarette smoke contains thousands of potentially harmful chemicals of which more than 40, including certain polycyclic aromatic hydrocarbons, nitrosamines and aldehydes, have demonstrated carcinogenic activity in vivo or in vitro (Clemens, 1991). High concentrations of free radicals are present in both the aqueous and the tar phase of cigarettes (10^{14}–10^{15} radicals in each puff of smoke; Church and Pryor, 1985; Halliwell and Gutteridge, 1989), and they are strongly implicated in smoking-related diseases. It is estimated that as many as 40% of all cancers are due to cigarette smoking (Doll and Peto, 1981). Radicals and metals found in cigarettes can react with oxygen, resulting in the formation of the superoxide radical, hydrogen peroxide, and ultimately the hydroxyl radical (Cosgrove et al., 1985). Reactive oxygen species, found in aqueous extracts of cigarette tar, are implicated in genotoxicity, altering DNA bases (Cross et al., 1987), causing chromosomal abnormalities in the form of sister-chromatid exchanges in human lymphocytes (Hopkins and Evans, 1979) and inducing DNA single-strand breaks in both bacteriophage DNA and DNA from cultured human lung cells (Nakayama et al., 1985; Cross et al., 1987; Willey et al., 1987). In spite of this evidence for genotoxicity, investigations of *hprt* mutagenesis in smokers have not yielded consistent results. Smokers in our study had a 1.5-fold elevation in *hprt*$^-$ mutant frequency compared with non-smoking individuals of a similar age, in good agreement with data from Cole's laboratory showing a 56% increase in *hprt*$^-$ mutant frequency with smoking (Cole et al., 1988,1990). Other laboratories have reported an increase in mutant frequency with smoking (Branda et al., 1991; Caggana et al., 1991; Tates et al., 1991) while some studies have found no correlation or even an inverse relationship (Sala-Trepat et al., 1990; Davies et al., 1992; Branda et al., 1992,1993). The association between smoking and mutant frequency is strengthened by our finding of a significant positive correlation between *hprt*$^-$ mutant frequency and number of cigarettes reported to be smoked daily. To our knowledge this is the first time such a correlation has been demonstrated, which may be a result of the stringency with which smokers and non-smokers were recruited in our study.

There is considerable evidence from both experimental and epidemiological studies that consumption of fruits and vegetables, high in antioxidants, reduces both DNA damage and the risk of cancer (Negri et al., 1991; Simic and Bergtold, 1991; Steinmetz and Potter, 1991; Block, 1992; Gerster, 1993). We have investigated the association between plasma concentrations of some of the most important dietary antioxidants and the frequency of mutation at the *hprt* marker gene. Vitamin C, the most abundant water-soluble antioxidant present in the body, in addition to its ability to deactivate potentially carcinogenic nitrites in the stomach, is a powerful free-radical scavenger. Low plasma levels and dietary intakes of vitamin C are associated with a high incidence of cancer and DNA damage, while supplementation protects against endogenously generated oxidative base damage (Block, 1991; Knekt et al., 1991a; Stahelin et al., 1991; Fraga et al., 1991). Vitamin C also reduces UV or chemically induced tumour formation in animals (Byers and Perry, 1992). Vitamin E, the most significant fat-soluble chain-breaking antioxidant found in lipid membranes, is associated with a reduced risk of certain human cancers (Dorgan and Schatzkin, 1991; Knekt et al., 1991a,b; Stahelin et al., 1991) and experimentally induced animal tumours (Hayatsu et al., 1988; Byers and Perry, 1992). In addition to provitamin A activity, several of the lipid-soluble carotenoid pigments are antioxidant in nature, acting either as free radical scavengers or as singlet oxygen quenchers.

Numerous epidemiological trials have shown β-carotene, the most extensively studied of the carotenoids, to be protective against a variety of human malignancies, most notably lung cancer

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(Connet et al., 1989; Ziegler, 1989; Dorgan and Schatzkin, 1991), although a recent trial carried out in Finland has found that giving β-carotene prophylactically significantly increases lung cancer risk (The Alphatocopherol, Betacarotene Cancer Prevention Study Group, 1994). Animal and in vitro studies have shown β-carotene to inhibit chemical-induced DNA damage (Hayatsu et al., 1988; Byers and Perry, 1992; Rousseau et al., 1992; Bertram et al., 1991).

In our present study, low plasma ascorbic acid levels were weakly associated with a high frequency of *hprt*⁻ lymphocytes in the population as a whole. Since smokers have markedly lower vitamin C levels and higher *hprt*⁻ mutant frequencies compared with non-smokers, such an association is not surprising. However, within the group of smokers alone, there is a rather strong negative correlation between mutant frquency and vitamin C level, suggesting that there may in fact be a protective effect of vitamin C. It is conceivable that different patterns of vitamin C intake in different populations might partly account for the lack of consistency among studies reflecting smoking with *hprt*⁻ mutant frequency. None of the carotenoids showed any significant association with *hprt*⁻ mutant frequency. Plasma levels of antioxidants reflect intake of fruit and vegetables, and this particular geographical area (Scotland) traditionally has a low consumption of such foods (Gregory et al., 1990; The Scottish Diet, 1993). Therefore, a comparative study of a population with a considerably higher fruit and vegetable component in the diet may be illuminating. However, the large number of variables which can influence the *hprt* lymphocyte cloning assay makes interpretation of data gathered from two distinct populations difficult. A different approach is to examine the spectrum of *hprt*⁻ mutations in different populations and to relate the degree of oxidative damage (inferred from the types of mutation seen) with plasma antioxidant levels. This is currently under way in our laboratory.

In conclusion, in this study *hprt*⁻ mutant frequency was elevated in smokers compared with non-smokers and showed a positive relationship with cigarette consumption. *Hprt*⁻ mutant frequency was higher in individuals with low plasma vitamin C levels suggesting that dietary factors may be protective against DNA damage at this site. However, low plasma vitamin C levels and cigarette smoking are intimately linked, making estimation of the relative importance of these variables on *hprt*⁻ mutant frequency difficult.

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